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Multi-scale Observation of Biological Interactions of Nanocarriers: from Nano to Macro

Su-Eon Jin, Jin Woo Bae, and Seungpyo Hong*

Department of Biopharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago, Chicago, IL 60612

Abstract

Microscopic observations have played a key role in recent advancements in nanotechnology-based biomedical sciences. In particular, multi-scale observation is necessary to fully understand the nano-bio interfaces where a large amount of unprecedented phenomena have been reported. This review describes how to address the physicochemical and biological interactions of nanocarriers within the biological environments using microscopic tools. The imaging techniques are categorized based on the size scale of detection. For observation of the nano-scale biological interactions of nanocarriers, we discuss atomic force microscopy (AFM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). For the micro to macro-scale (*in vitro* and *in vivo*) observation, we focus on confocal laser scanning microscopy (CLSM) as well as *in vivo* imaging systems such as magnetic resonance imaging (MRI), superconducting quantum interference devices (SQUIDs), and IVIS®.

Additionally, recently developed combined techniques such as AFM-CLSM, correlative Light and Electron Microscopy (CLEM), and SEM-spectroscopy are also discussed. In this review, we describe how each technique helps elucidate certain physicochemical and biological activities of nanocarriers such as dendrimers, polymers, liposomes, and polymeric/inorganic nanoparticles, thus providing a toolbox for bioengineers, pharmaceutical scientists, biologists, and research clinicians.

Keywords

nanocarriers; microscopy; multi-scale observation; nano-bio interactions

INTRODUCTION

National Nanotechnology Initiative (NNI, www.nano.gov) defines nanotechnology as “the understanding and control of matter at dimensions between approximately 1 and 100 nanometers, where unique phenomena enable novel applications. Encompassing nano-scale science, engineering, and technology, nanotechnology involves imaging, measuring, modeling, and manipulating matter at this length scale.” Nanomaterials (materials with at least one dimension between 1 and 100 nm) have unprecedentedly large surface-to-volume ratios that are directly related to a variety of unique physical, chemical, and biological properties of the materials (Nel et al., 2009). In particular, as one of the promising applications of nanotechnology is utilization of nanomaterials as nanocarriers (as the same terminology used by Peer *et al.* (Peer et al., 2007)), appropriate and complete identification of surfaces of nanomaterials with biological substances, i.e. *nano-bio interface*, is critical to

*To whom all correspondence should be addressed: Department of Biopharmaceutical Sciences, College of Pharmacy University of Illinois at Chicago 833 S. Wood St. Rm 335, Chicago, IL 60612 Phone: (312) 413-8294 Fax: (312) 996-0098 sphong@uic.edu .

ultimately develop highly specific, controllable, and effective nanocarriers (Nel et al., 2009; Torchilin, 2006). The biological interactions of nanocarriers occur, at the multi-scale, with counterparts such as DNA/RNA (a few angstroms to a few nanometers), proteins (a few to tens of nanometers), lipid cellular membranes (tens to hundreds of nanometers), cells (a few to tens of micrometers), and animals and humans (a few to hundreds of centimeters). Among many exciting evolutions in nanotechnology, recent advances in minimally invasive imaging techniques allow scientists to continue to hold the catchphrase “seeing is believing” over the wide size range. This review covers high-resolution imaging techniques such as atomic force microscopy (AFM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM) to *in vitro* and *in vivo* imaging using confocal laser scanning microscopy (CLSM), magnetic resonance imaging (MRI), superconducting quantum interference device (SQUID), and IVIS®. Characteristics of each technique with exemplary references are listed in Table 1. Additionally, recently developed combinations of multiple microscopic technologies to achieve simultaneous multiple-scale imaging are also summarized. Figure 1 summarizes nano-bio substances at the multiple size scales with a list of imaging techniques that cover the size ranges, offering an overview of this review.

MULTI-SCALE OBSERVATION OF NANO-BIO INTERFACES

1. Microscopic tools for the nano-scale observation

High-resolution imaging technologies enable characterization and manipulation of materials at the nano-level and further elucidate nano-scale phenomena. Without such technologies, promising nanomedicines with great potential for diagnoses and treatments of debilitating diseases may have not been developed. This section summarizes the three most commonly used high-resolution imaging techniques – AFM, SEM, and TEM.

1.1. Atomic force microscopy (AFM)—AFM is a representing member of scanning probe microscopes (SPMs) that generate images of specimens by ‘feeling’ rather than by ‘looking’, which is the unique aspect that clearly distinguishes AFM from other high-resolution techniques such as electron microscopes (EMs) (Morris, 1999). Unlike EMs that require pre- or post-treatment of samples, AFM is capable of atomic resolution of flat surfaces and such resolution can be achieved in both gaseous and liquid (thus physiologically relevant) environments without any additional treatments to the specimens. This versatile technique was invented by the Nobel laureates Gerd Binnig and Heinrich Rohrer, with co-inventors Calvin Quate and Christoph Gerber, in 1986 (Binnig et al., 1986), of which the basic principle is illustrated in Figure 2a. Since then, a large number of studies have used this revolutionary method – a Web of Science search with keywords of ‘atomic force microscopy’ gives approximately 50,000 publications to date (for extensive reviews, see (Carpick and Salmeron, 1997; Niemeyer, 2001)).

AFM is able to image a broad size-range, covering from the atomic scale to hundreds of micrometers. For nanomaterials and biomolecules, atomic resolution is only possible for simple molecules in which each atom is in intimate contact with the surface of a flat substrate – typically in the gaseous phase. For example, AFM was employed to image individual 3-10 nm poly(amidoamine) (PAMAM) dendrimer macromolecules (Mecke et al., 2004a) and dendrimer nanoclusters conjugated through DNA (10-20 nm in length) (Choi et al., 2004), on a flat mica surface. AFM-based visualization of other types of nanocarriers, such as liposomes (50-500 nm) (Ruozi et al., 2007), polymeric nanoparticles (50-200 nm) (Dong and Feng, 2004), carbon nanotubes (Li et al., 2001), and inorganic nanoparticles (gold nanoparticles (Zharov et al., 2005), iron oxide nanoparticles (Ma et al., 2007), and nanodiamonds (5-30 nm) (Huang et al., 2008b)), and their biological interactions have been extensively studied.

AFM also allows sub-molecular resolution on most biological substances under physiological aqueous conditions, which makes AFM an ideal tool for biological settings. The Banaszak Holl group at the University of Michigan reported a series of papers regarding nano-scale nanoparticle-membrane interactions with supported lipid bilayers, directly observed by AFM (Leroueil et al., 2008; Mecke et al., 2005b; Mecke et al., 2004b). The positively charged nanoparticles, both synthetic and natural, including PAMAM dendrimers (Hong et al., 2004), cationic linear polymers such as poly-L-lysine, polyethylenimine, and diethylaminoethyl-dextran (Hong et al., 2006a), cell penetrating peptides (CPPs; MSI-78) (Mecke et al., 2005a), proteins (TAT), gold nanoparticles (Au-NH₂), and silicon oxide nanoparticles (SiO₂-NH₂) (Leroueil et al., 2008) all induce formation of nano-scale holes (10-40 nm in diameter), membrane thinning, and/or membrane erosion. Figure 2b, c, and d show a set of AFM images monitoring nano-bio interactions between dendrimers and supported lipid bilayers (reproduced with copyright permission by American Chemical Society, license number 2347960600330). The AFM results were further validated *in vitro*, suggesting that the nano-scale hole formation mechanism plays an important role in cellular internalization of this class of nanomaterial (Hong et al., 2004; Hong et al., 2006a). This nano-scale hole formation at least partially explains the mechanism of gene (plasmid DNA or siRNA) delivery mediated by cationic nanocarriers (Hong et al.; Leroueil et al., 2007).

In addition to the atomic resolution imaging capacity, AFM also measures nano-scale forces, which is particularly useful for detection of an extremely weak force that is generated as a result of nano-bio interactions (Dufrene, 2003). The direct measurements of an extremely small amount of force (e.g. forces exerted by a single live cell) are enabled by two AFM-based approaches: real time cellular force spectroscopy (CFS) and functionalized force imaging (FFI). Real-time CFS measures the deflection of cantilevered probes engaged at the cell surface, and FFI quantifies the position of single molecules on sample surfaces via specific interactions between molecules on the probes and the surfaces. Using the method, Lee *et al.* measured binding kinetics between cell surface receptors and extracellular biomolecules that were coated on the AFM tip, along with force-based visualization of receptor distribution on the cell surface (Lee et al., 2007b).

Although highly versatile and powerful, AFM suffers from a few disadvantages that often require combined studies with other supplementary techniques. Due to the extremely small area of interaction between the AFM tip and the specimen surface, it is hard to achieve a population study of a number of cells and samples that is, in most cases, important to the nano-scale monitoring in understanding unbiased nano-bio interactions. Further, since images (or forces) measured with an AFM are always a convolution of the probe geometry and the shape of the features being imaged and atomic sensitivity of piezoelectricity, a variety of potential artifacts can be induced (Alcaraz, 2002; Westra, 1993). The image artifacts are typically caused by types, sizes, and angles of probes, edge overshoot and drift of scanners, errors during image processing, floor and acoustic vibrations, and other sources such as surface contamination and vacuum leaks.

1.2. Scanning electron microscopy (SEM)—SEM has been generally used in observation and characterization of solid materials from the nano- to micro-scale, coupled with appropriate methods of sample coating using conducting metallic compounds such as gold and platinum (Goldstein, 2003). An SEM image is generated through the energy transfer between the scanned electron beam and the emitted electron from a specimen. Figure 3a shows that the principle of SEM analysis that detects the secondary electron and the X-ray emitted from the sample, which are scanned by electron beam, resulting in a three-dimensional image. An example of poly(lactic-co-glycolic acid) (PLGA) microparticles is shown in Figure 3b. One of the advantages of this technique, especially compared to AFM, is that this imaging technique eliminates the potential artifacts that AFM often has since the

SEM images are not taken based on physical interactions between the probe and the surface of a specimen.

The high-resolution imaging technique has been widely applied for determination of surface morphology of solid-state materials including cells, tissues, intracellular surfaces, viruses, proteins, and nucleic acids (Drummond and Allen, 2008; Goldberg, 2008). Intermolecular and biological interactions of nanocarriers can be also observed by SEM (Huang et al., 2008a; Kim et al., 2009a; Nel et al., 2009; Srinivasan et al., 2009). The three-dimensional SEM images provide great details on morphological and physical properties of nanocarriers such as carbon nanotubes (Day et al., 2005), PLGA nanoparticles (Win and Feng, 2005), and inorganic nanoparticles including gold nanoshell (Nehl et al., 2004) and silica nanoparticles (Fleming et al., 2001). For example, SEM observations have been used to collect information regarding those nanocarriers in terms of size, shape, porosity, and release kinetics of incorporated bioactives by morphological changes. Further, cellular uptake of nanocarriers can be also monitored by SEM although *in situ* observation is generally not possible due to the additionally required coating steps onto the cell samples. As an example of the SEM-based studies, supraparamagnetic iron oxide nanoparticles with and without poly(ethylene glycol) (PEG) coating were compared after incubation with human fibroblasts (Gupta and Curtis, 2004). From the SEM images, morphological changes in the cells indicated that the PEGylated iron oxide nanoparticles internalize into the cells without causing major damage whereas the non-PEGylated nanoparticles induce significant damage to the cell membrane.

One drawback of this technique is that the SEM specimens need to be coated by a conducting material with thickness of 20-30 nm to enable the electron-based detection. The coating process increases the sample preparation steps that may cause damage to the specimens, preventing *in situ* observations of the nano-bio interactions. Since the images can be taken only in the dry phase, observation of the nano-bio interactions in physiological wet conditions is often limited. This disadvantage has been somewhat addressed by recent advances in the technique. The cryo-technique enables to image soft materials such as liposomes that are technically difficult to be observed using the standard SEM (Goldstein, 2003; Lee et al., 2007a; Nel et al., 2009). Low-voltage SEMs can provide high contrast images without the sample staining steps (Drummy et al., 2004; Ilona et al., 2003), and a wet SEM performs under the wet condition (Nyska et al., 2006).

1.3. Transmission electron microscopy (TEM)—TEM has been utilized for analysis of inner micro/nano-scale structure, crystallinity, and chemical components of nanomaterials (Goldberg, 2008). Basically, a TEM image is two-dimensional and is formed by scattered electrons produced by an electron beam flood onto a sample, as demonstrated in Figure 3c. An example of TEM images is shown in Figure 3d – polymeric nanoparticles prepared from PEG-*b*-poly(ϵ -caprolactone) copolymer via a nanoprecipitation method. The sub-nanometer resolution of TEM provides information on inner-particle structures of the nanocarriers, which confirms, for example, encapsulation of bioactive materials inside the nanocarriers (Dilag et al., 2009; Kim et al., 2008). Moreover, by observing cellular uptake of nanocarriers, TEM can be used to elucidate interactions of nanocarriers with biological macromolecules, e.g. gold nanoparticles (40 nm) with oligonucleotides or DNA in cells (Lee et al., 2006b; Xu et al., 2006; Zharov et al., 2005). The resolution of TEM can be improved up to 0.1 nm using z-contrast scanning transmission electron microscopy (STEM), electron energy loss spectroscopy (EELS), and high resolution TEM (HRTEM) (Ernst and Ruhle, 1997).

One of the drawbacks of TEM is that the ideal specimen thickness should be less than 100 nm to obtain high-resolution TEM images because electron energy cannot pass beyond a

few hundred nanometers. Due to the limitation of sample thickness, additional sample preparation steps using jet polishing, microtomy, ion milling, or focused ion beam (FIB) (Giannuzzi and Stevie, 1999; Pantel et al., 1997) are required. Further, as the images are typically taken in the dry condition, there are limitations in imaging nanocarriers in biological environments.

2. Microscopic tools for the micro- and macro-scale observation

Although high-resolution imaging techniques have played a leading role in understanding phenomena occurring at the nano-bio interfaces, imaging a relatively large area (from a few micrometers to the whole body-scale of animals and humans) is equally important, especially for successful clinical implementation of nanotechnology. Nanocarriers for the delivery of bioactive and imaging agents have been monitored by micro- and macro-scale imaging technologies to elucidate: i) delivery potential to target sites *in vivo*; ii) modulation of cellular functions after cellular uptake of nanocarriers; and iii) molecular and physiological changes at various disease stages. In this section, we review CLSM (arguably the most powerful microscope to observe cells and tissues), MRI and more advanced SQUIDS-detected MRI (the high-resolution *in vivo* imaging tool), and IVIS® (the bioluminescence detection with the whole-body scan capability for small animals). Figure 1 also demonstrates the corresponding size scales for each technique. Although not discussed in detail in this review, other important techniques include X-ray fluorescence microscopy (XFM) and optical coherence tomography (OCT). XFM reveals intracellular distribution of trace elements of interest, providing quantitative elemental maps at a submicrometer spatial resolution in cell and tissue samples (Kehr et al., 2009). OCT is an optical signal acquisition and processing method that provides micrometer-resolution three-dimensional images of biological tissues. As this technique employs long-wavelength near-infrared light that penetrates deeply into the scattered medium, the deeper internal microstructures (up to ~2 mm from the surface) of tissues can be visualized, which is significantly enhanced from the depth resolution of confocal microscopy (Huang et al., 1991; Licha and Olbrich, 2005).

2.1. Confocal laser scanning microscopy (CLSM)—CLSM is a technique that obtains in-focus images with high resolution (lateral, 140 nm; axial, 1 μ m) and depth selectivity based on detection of fluorescence from a specimen (Alvarez-Rom et al., 2004). CLSM only collects the in-focus light that passes a pinhole from the reflected light of laser by a photodetection device (commonly a photomultiplier tube (PMT) or avalanche photodiode), in which process detection of the out-of-focus light is substantially suppressed. Thus, the laser passes through scanning optics, reaches the sample, and accurately defines an image of a given focal plane, which permits obtaining images of planes at various depths within the sample (sets of such images are also known as ‘*z stacks*’). This is a major advantage of CLSM allowing optical *z* sectioning with significantly enhanced image sharpness when compared to the conventional fluorescence microscope.

CLSM offers one of the most powerful tools in visualizing the nano-bio interfaces with direct involvement of living organisms such as cells and tissues. The recent advances in nanotechnology-based fluorophores enable this imaging technique to be even more versatile in numerous biological applications (White and Errington, 2005). For instance, CLSM has been widely used to understand cellular uptake mechanisms of nanocarriers such as dendrimer, liposome, and nanoparticle by observing co-localization with a variety of endocytic markers (Hong et al., 2009; Sugano et al., 2000; Verma et al., 2008). The endocytic markers stain specific cellular components and/or compartments that are known to play an important role in certain cellular internalization mechanism, e.g. transferrin (Tf) for clathrin-dependent pathways and cholera toxin subunit B (CTB) for lipid raft-mediated endocytosis, among others (Foerg et al., 2005; Richard et al., 2003). Hong *et al.* (Hong et al.,

2009) employed AlexaFluor® 488-tagged PAMAM dendrimers with various sizes and surface charges, and observed intracellular locations of the nanocarriers using CLSM. To explore the nano-bio interactions, cells were observed after co-incubation with the dendrimer nanocarriers and the endocytic markers at 37 °C, allowing endocytosis and/or pinocytosis, and at 4 °C to prohibit the energy-dependent mechanisms. The CLSM observation of dendrimer internalization even at 4 °C and inconclusive co-localization with Tf or CTB, combined with the results using other methods including AFM as described above (Hong et al., 2004; Hong et al., 2006b), suggest that the nano-scale hole formation mechanism should be considered as an operating mechanism of dendrimer entry into the cells (Hong et al., 2009).

For *in vivo* tumor models, the interactions of nanocarriers and tumor tissue were observed using CLSM, confirming that certain-size nanocarriers accumulate in tumor tissue by the enhanced permeation and retention (EPR) effect (Liu et al., 2009; Zhu et al., 2009). Recent progress in CLSM also allows *in vivo* study without mechanical sectioning and fixation. A non-invasive reflectance CLSM method is developed for *in vivo* biomedical applications of diagnostics at topical and local tissues (e.g. eye, oral cavity, and skin) although it has a limitation in the use of deep tissues (Chiou et al.; Maitland et al., 2008; Nehal et al., 2008).

2.2. Magnetic resonance imaging (MRI)—MRI is a powerful *in vivo* microscopic imaging technique that provides biochemical and physiological information, without radiolabeling, in the presence of a magnetic field (Kozłowska et al., 2009; Mody et al., 2009). In diagnostics, MRI has been utilized as a sensitive detection method for soft tissue physiology and pathology in various diseases, including thrombolysis, inflammatory diseases, neurodegeneration, and cancer (Rudin and Weissleder, 2003). MR images are obtained from the transition of energy levels of protons in tissue, based on nuclear spin reorientation of protons in an applied magnetic field. In principle, protons in the applied magnetic field are aligned, followed by returning to the original state – this is called relaxation, consisting of longitudinal relaxation (T_1 ; recovery) and transverse relaxation (T_2 ; decay) to generate an MR image. The commonly used MRI systems in clinics use relatively low magnetic field strengths (1.5-3 tesla (T)), resulting in a limited resolution of the 2-3 mm range. At higher magnetic field strengths in the laboratory-scale, resolution of MR images can be improved to be in the range of 10-100 μm (Brindle, 2008). For the high resolution, however, image acquisition typically takes from several minutes to a few hours, which is much longer than the millisecond range of other fast imaging techniques, including computed tomography (CT), fluorescence reflectance imaging (FRI), and ultrasound.

The MRI technique using magnetic nanoparticles (MNPs) that can be used as contrast agents for disease diagnosis as well as delivery vehicles of drugs has undergone rapid development (McCarthy and Weissleder, 2008; Sun et al., 2008). MNPs can enhance the contrast of an MR image by shortening relaxation steps of surrounding protons (relaxivity) by increased local concentrations of MNPs in tissue. Recently developed MNPs include metallic nanoparticles (mostly iron oxide nanoparticles (Peng et al., 2008)) and modified iron oxide nanoparticles by surface coating with liposomes/lipid micelles (Kozłowska et al., 2009; Torchilin, 2006), polymers (Lee et al., 2006a), and dendrimers (Mody et al., 2009). Those modifications of MNPs are to reduce toxicity of the metallic compounds while enhancing stability and tissue uptake at target tissue for high-resolution images. It is reported that PEG-coated iron oxide nanoparticles improve solubility and stability of the nanoparticles in biological environments as well as prolong the circulation time of the contrast agents (Lee et al., 2006a). Furthermore, other biocompatible polymers such as hyaluronic acid and poloxamer derivatives have been used to coat iron oxide nanoparticle (Kumar et al., 2007; Lee et al., 2006a). Superparamagnetic iron oxide nanoparticle (SPION, 60-150 nm in diameter) and ultrasmall SPION (USPION, 10-50 nm) with targeting ligands have been

developed with demonstrated *in vivo* efficacy due to their relatively small size (5-150 nm) and high potential of magnetization that is required for *in vivo* biomedical imaging [for extensive review, see (Peng et al., 2008)]. Liposomes and lipid-based micelles also have attracted much attention because of their pharmaceutical characteristics such as easily controlled size and biocompatibility. Liposomes (70-200 nm) can carry contrast agents in the aqueous inner core and diagnostic moieties (e.g. PAP) in the membrane bilayers, and lipid-based micelles formed via self-assembly can also be similarly used (Torchilin, 2000). Additionally, dendrimers (1-10 nm) have been used for delivery of contrast agents with targeting moieties using various surface conjugation chemistries through available primary amine groups (Landmark et al., 2008; Mody et al., 2009).

2.3. Superconducting quantum interference devices (SQUIDs)-Detected MRI—

The very sensitive SQUIDs that measure extremely weak magnetic fields as low as 5 aT (5×10^{-18} T), based on superconducting loops containing Josephson junctions (Mahdi and Mapps, 1998; Ran, 2004), are a promising technique for high-resolution imaging of MNPs. SQUIDs are being used as detectors to perform MRI. While high-field MRI uses precession fields of one to several T, SQUID-detected MRI typically uses the fields in the μ T regime. This technique overcomes limited resolution of MRI by the sensitivity loss of Faraday detection at low frequencies, allowing high-resolution SQUID-detected MRI, as demonstrated by the 10-mT system (Seton et al., 1997) and by the 132 μ T superconducting untuned input circuit (McDermott et al., 2004).

Examples of biological applications of SQUIDs include magnetoencephalography (MEG), use of an array of SQUIDs to make inferences about neural activity inside brains (Kakigi et al., 2000; Vrba and Robinson, 2002), and magnetogastrography (MGG), recording of the weak magnetic fields of the stomach (Irimia and Bradshaw, 2005). Recently, Ge *et al.* reported a remanence measurement method to detect trace amounts of iron oxide nanoparticles, achieving the *in vivo* detection sensitivity of 10 ng of 25 nm Fe₂O₃ nanoparticles at a distance of 1.7 cm with the spatial resolution of $\sim$ 1 cm, offering an alternative *in vivo* imaging technique (Ge et al., 2009). Moreover, with the improved quality of MNPs that have enhanced specificity and sensitivity towards target cells/tissues (Shi et al., 2008; Wang et al., 2007), this non-invasive imaging technique can be armed with high bio-specificity, becoming ideal for biological applications, e.g. early detection of cancer and monitoring of cancer progress upon treatments. For instance, breast tumors are well resolved in high-field MRI, but the technique is too expensive for serial imaging, which is required for effective prognosis. Prostate tumors are resolved in T2-weighted images (Claus et al., 2004) that offer useful resolution, but this method is also too costly to be implemented for routine use. Therefore, if a relatively inexpensive SQUID-detected MRI system empowered by advanced MNPs can be implemented to image tumors, it could have a significant impact on cancer diagnosis and prognosis.

2.4. IVIS® Imaging Systems—

IVIS® (Xenogen, Caliper Life Sciences, Hopkinton, MA) is an optical molecular imaging device with charge coupled device (CCD) camera for a non-invasive, live imaging system from analysis of bioluminescence (Weissleder and Pittet, 2008). For the high-resolution in IVIS®, a cooling method of CCD is used to improve the sensitivity and to inhibit the noise from thermal energy at room temperature (Contag et al., 1998). With temperature decrease in CCD by 20 °C, it is reported that imaging sensitivity is enhanced by 10% due to noise rate reduction (Meyer and Kirkland, 2000). As IVIS® can detect luminescence and fluorescence with relatively high sensitivity, it has been widely used for *in vivo* visualization of small animals (Contag et al., 1998; Weissleder, 2001). It has been applied for real time imaging of gene expression, cellular trafficking, and molecular interactions between cells and microstructure in live tumor models, without employing contrast agents such as MNPs for MRI and SQUIDs (Weissleder and Pittet, 2008). In

addition, the *in vivo* fate of nanocarriers during drug delivery (e.g. biodistribution and accumulation of nanocarriers) can be monitored using this technique without sacrificing animals. This technique would be particularly useful, when combined with the development of fluorophore-tagged nanocarriers for delivery of therapeutics such as chemotherapeutic drugs, peptides, DNA (Kim et al., 2009b), and siRNA (Yagi et al., 2009).

3. Combined Systems of Multiple Imaging Technologies

3.1. Combined AFM-CLSM—As described above, AFM offers nano-scale resolution in surface morphology and force mapping, enabling direct observation of the nano-scale behaviors of materials of interest. On the other hand, CLSM provides spatial resolution in a range of hundreds of nanometers to hundreds of micrometers with an excellent capability of 3-dimensional imaging by its high z-directional resolution. Combination of AFM and CLSM (similarly also with a conventional epifluorescence microscope) thus provides a powerful tool that compensates strengths and weaknesses of the two techniques, which is ideal for the investigation of nano-bio interactions (Haupt et al., 2006; Mangold et al., 2008; Owen et al., 2006). This combined microscopy has a distinct advantage of the live cell imaging capacity without further treatments on the biological specimens. Javier *et al.* reported the use of an AFM/optical microscope to investigate relationship between the adhesion forces (measured by AFM) and the uptake rate (observed by the light microscope) (Munoz Javier et al., 2006). By employing polymer microparticles that are surface-coated with positively charged poly(allylamine hydrochloride) and negatively charged poly(styrenesulfonate), they found that strong adhesion between cationic microspheres and cells resulted in rapid cellular uptake. In addition to the visualization capacity, combination of AFM and CLSM can provide a multifunctional imaging station that is capable of: i) Nano- to pico-Newton-scale force measurement; ii) Förster resonance energy transfer (FRET); iii) Total internal reflection fluorescence (TIRF); and iv) Fluorescence recovery after photobleaching (FRAP).

3.2. Correlative Light and Electron Microscopy (CLEM)—Although light microscopes can possibly resolve only up to approximately 200 nm according to the resolution equation, recent studies demonstrate that fluorescence microscopy has been developed into “nanoscopy” that can resolve on the order of 50-100 nm (Gustafsson, 2008). Further, near-atomic level resolution can be introduced by combining the advanced nanoscopy with EMs that offer 3-dimensional tomography (Hoenger and McIntosh, 2009), particularly in the case of observing phenomena at the nano-bio interface at the cost of sacrificing live cell imaging. To circumvent the limitation of imaging live cells, a new tool is presented that allows live viewing a sample under the light microscope and rapidly freeze this same sample within 5 seconds by high pressure freezing (Sartori et al., 2007). For the recent advancements and perspectives of the CLEM technique, readers are referred to an extensive review by Cortese *et al.* (Cortese et al., 2009).

3.3. Combined SEM-spectroscopy—Recently, combined microscopic techniques between SEM and spectroscopy have been introduced to simultaneously acquire SEM images along with chemical, physical, and structural analysis and quantification. Examples include combinations of SEM X-ray spectroscopy (Sayen et al., 2009) and ¹H-NMR spectroscopy (Strübing et al., 2007). SEM combined with X-ray spectroscopy measures marker elements for tissue analysis, which is useful for the determination of the function of elements in physiological conditions and for disease diagnosis (Gupta and Hall, 1981; Thakral and Abraham, 2007). Combined with ¹H-NMR spectroscopy, SEM reveals film morphology as well as compositions that quantify release kinetics of the polymeric coating agents in the film of coated oral dosage forms (Strübing et al., 2007).

SUMMARY

Over the past decade, nanotechnology has affected numerous scientific fields and industry. Perhaps arguably, the most promising application of nanotechnology to date is personalized, disease-specific nanomedicine using nanocarriers. However, at the same time, there are emerging concerns regarding toxicity of nanomaterials, which has resulted in a new discipline called ‘nanotoxicology’. Here we have discussed commonly used imaging techniques that have various resolution scales. To completely understand the nano-bio interactions, multiple-scale observation spanning from the nano- to macro-scale of a given phenomenon is necessary. Only complete understanding on the nano-bio interfaces will enable us to have full control over biological properties of the nanocarriers, which will help to eliminate the recently raised toxicity concerns. Being equipped with powerful microscopic techniques, we are moving positively towards curing debilitating diseases such as cancer using nanotechnology that is safe and yet highly effective.

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Figure 1.

Scale-based representative objects and corresponding microscopic tools. (a) H₂O, water molecule (~2 Å), (b) DNA double helix (2-10 nm), (c) dendrimer (1-10 nm), (d) liposome (50-500 nm), (e) nanoparticle (50-200 nm), (f) cells (5-50 μm), and (g) a mouse (2-10 cm).

Figure 2.

(a) Schematic diagram of an AFM (adapted with modification, from Alessandrini and Facci, *Meas. Sci. Technol.* 2005, 16, R65–R92). An AFM images surfaces or measures forces by scanning a tiny tip mounted at the end of the cantilever on a sample, which is controlled by a piezoelectric scanner. Interactions between samples and the tip are recorded by laser that is reflected to a split photodetector. (b)–(d) AFM height images of DMPC bilayer after interaction with dendrimers (reproduced by copyright permission by American Chemical Society, license number 2347960600330). White lines highlight some of the areas where dendrimers have affected the bilayer (the dendrimer themselves cannot be seen on these images). (b) Generation 7 PAMAM dendrimers (G7-NH₂) cause the formation of small holes, 15–40 nm in diameter, in the previously intact parts of the bilayer (see dark spots). (c) G5-NH₂ remove lipid molecules primarily from edges of existing bilayer defects, i.e., dark areas grow in size, see arrows. (d) Acetylated G5 dendrimers (charge neutral) do not remove lipid molecules from the surface. Instead they adsorb to edges of existing bilayer defects.

Figure 3. Principles and example images of SEM and TEM. (a) Principle feature of SEM and (b) an SEM image of poly(lactide-*co*-glycolide) microparticles prepared by a double emulsion method. (c) Principle feature of TEM and (d) a TEM image of polyethylene glycol-*b*-poly(ϵ -caprolactone) nanoparticles prepared by a nanoprecipitation method.

Table 1

Summary of representative microscopic techniques

Microscopy	Description & application	Size scale	Characteristics	Ref.
<i>Nano-scale observation</i>				
AFM	Atomic force microscopy, one of scanning probe microscopy, useful for studies on surface morphology and size of nanocarriers; interactions of nanocarriers in biological environment.	1-10 Å: observed small molecules 1-10 nm: dendrimers 50-200 nm: nanoparticles 50-500 nm: liposomes 0.5-50 µm: microparticles 5-500 µm: cell-cell, cell-peptide, cell-nanocarriers	Nano-scale topology and biological interaction (force mapping)	Alessandrini and Facci, 2005; Huang et al., 2008b; Mecke et al., 2004a; Ruozzi et al., 2007)
SEM	Scanning electron microscopy, one of electron microscopy, used for surface of nanoparticles and applied for interaction of protein, polyplex or lipoplex with cell surface using high-resolution SEMs.	50-200 nm: nanoparticles 50-500 nm: liposomes 0.5-50 µm: microparticles 5-500 µm: cell-cell, cell-peptide, cell-nanocarriers	3-D type images Requires coating with conducting materials	Drummond and Allen, 2008; Goldberg, 2008; Srinivasan et al., 2009)
TEM	Transmission electron microscopy, one of electron microscopy, utilized for determination of inner core structure of nanocarriers.	50-200 nm: nanoparticles 50-500 nm: liposomes 0.5-50 µm: microparticles 5-500 µm: cell-cell, cell-peptide, cell-nanocarriers	2-D High resolution Requires preparation of samples as a thin layer (50-100 nm)	(Ernst and Ruhle, 1997; Giannuzzi and Stevie, 1999)
<i>Micro- and macro-scale observation</i>				
CLSM	Confocal laser scanning microscopy, using a near-infrared laser with low power of laser, applied for <i>in vitro</i> and <i>in vivo</i> imaging of cellular interaction and tissue distribution of nanocarriers.	5-100 µm: mostly observing cells (viable cells + fixed cells). 200-500 µm: preclinical imaging with fluorescent probes (attached with nanocarriers)	3-D by high magnification z-stacking Subcellular tracking Real-time histological diagnosis <i>in vivo</i> with limited depths and coverages	(Gerger et al., 2009; Hong et al., 2009; Verma et al., 2008)
MRI	Magnetic resonance imaging, useful for cellular uptake imaging without differentiation in environmental structures using gadolinium compounds, dysprosium, and iron oxide particles as a contrast agent.	Preclinical use: Rodent (2-10 cm) and larger animals (dogs and monkeys) Clinical use: anatomical, physiological, and molecular imaging in human	Diagnosis – intracellular monitoring and detection of disease (e.g. cancer) using contrast agents Cannot be used in patient with metallic device Long imaging time (hours)	(De et al., 2008; Landmark et al., 2008)
SQUIDS	Superconducting quantum interference devices, used as detectors to perform MRI to overcome limited resolution of MRI	<i>In vivo</i> , preclinical & clinical use	Enhanced specificity and sensitivity towards target cells/tissues with the improved quality of MNPs	(Shi et al., 2008; Wang et al., 2007)
IVIS®	Bioluminescence imaging device, applied for <i>in vivo</i> live animal imaging using luciferins combined with near-infrared fluorochrome for high-resolution.	<i>In vivo</i> , preclinical use in small animals	Molecular changes in live animals without artifact - gene expression and cell tracking Biodistribution - EPR effect of nanocarriers in <i>in vivo</i> tumor model	(Weissleder, 2001; Weissleder and Pittet, 2008; Yagi et al., 2009)